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THE FRAGILE X SYNDROME AND INFANTILE AUTISM: A PREVALENCE STUDY

Brian Herb Annex

1985

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The Fragile X Syndrome and Infantile Autism:
A Prevalence Study

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the degree of
Doctor of Medicine

by
Brian Herb Annex
1985

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I want to thank my parents and brother for all their love and devotion to me throughout the years. Finally to my loving wife Joellen, this work is dedicated to the first eight months of our marriage and what will be a happy life together.

Abstract

The Fragile X Syndrome and Infantile Autism:

A Prevalence Study

A thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine.

by Brian Herb Annex

Submitted: Febuary, 1985

The fragile X syndrome is a form of X-linked mental retardation that may be identified by special cytogenetic techniques. The fragile X syndrome is associated with mental retardation and macroorchidism, but other clinical features have been reported. Infantile autism is a syndrome of severe behavioral abnormalities that are manifested by 30 months of age. Infantile autism is frequently associated with mental retardation and is an etiologically heterogeneous disorder.

The fragile X syndrome has been implicated as the etiology for both the mental retardation and infantile autism in several males recently reported. In these reports there was speculation that the fragile X syndrome with features of infantile autism could explain part of the recognized male excess in infantile autism and that a high frequency of association between these disorders may exist.

This study was designed to determine the prevalence of the fragile X syndrome in a population of autistic mentally retarded males, since no studies of this type have been reported. Four of 47 males with infantile autism and mental retardation were found to have the fragile X syndrome giving a prevalence of 8.5%. This finding suggests that a significant proportion of autistic mentally retarded males may be affected by the fragile X syndrome. The significance of this finding and recommendations for the use of fragile X testing in males with infantile autism are discussed.

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CHAPTER ONE: Fragile X Syndrome

Part A: Nonspecific X-linked Mental Retardation

In 1938, Dr. L. S. Penrose [1] published the results of a large survey of the mentally retarded population in Colchester, England, in which he found significantly more affected males than females. As recently as 1963, he attributed this finding to a variety of social factors, not to any influence of the X chromosome [2]. This was the accepted theory for many years.

In 1972, Lehrke [3] also found an excess of mentally retarded males in his review of the literature and his own data derived from family studies of patients in the institutions for the mentally retarded in Wisconsin. Lehrke [3] postulated that X-linked genes were responsible for this male excess and perhaps a large proportion of all mental retardation. This hypothesis received strong criticism from authors [4,5] who continued to primarily use Penrose's [2] explanation. Lehrke [5], however, continued to defend his theory.

At the time of Lehrke's report [3] there were a number of reports published of large families in which mental retardation appeared to be inherited in an X-linked pattern. Among these reports were the families described by Martin & Bell [7] in 1943, and Renpenning et al. [8] in 1961. They discussed a form of X-linked mental retardation in which the affected persons had no

distinct physical abnormalities. This was termed nonspecific X-linked mental retardation.

Part B: Discovery of a Fragile X Chromosome

In 1969, Dr. Herbert A. Lubs, [9] then at the Yale University School of Medicine, noted a peculiar secondary constriction on the long arm of a C group chromosome in a significant percent of the lymphocytes in four mentally retarded males. They were all members of a family being studied cytogenetically because of a pattern of mental retardation consistent with X-linkage. By use of radioactive nucleotide labeling and evaluation of chromosomal size and arm ratios, Lubs concluded that this C group chromosome was the X chromosome. He went on to identify this secondary constriction in some of the obligate carriers. Dr. Lubs had identified a form of X-linked mental retardation with a cytogenetically identifiable abnormality on the X chromosome. He titled this paper, "A Marker X Chromosome."

Although many mentally retarded persons were being studied cytogenetically no similar cases of this marker chromosome were reported for a number of years. In 1976, Giraud et al. [10] published a report of 18 people with mental retardation and structural chromosomal variations. Six of these patients had identical chromosomal breaks at the distal end of the X chromosome. In 1977, Harvey et al. [11] also confirmed the initial studies of Lubs [9], with a report on a total of 14

mentally retarded males in four families with this X-chromosome abnormality.

In 1977, Sutherland [12,13] observed that special culture cell conditions were needed for reliable cytogenetic detection of this X-chromosome abnormaltiy, which he termed the fragile X chromosome. In 1979, Sutherland [14] defined a fragile site as a specific chromosomal point having four features: 1) it appeared as a nonstaining gap usually involving both chromatids; 2) it was located at an identical position between and within idividuals; 3) it showed Mendelian codominant inheritance; and 4) its fragility was exhibited as acentric fragments, deleted chromosomes, triradial formation, etc.. The terms, fragile X chromosome and marker X chromosome, are used by most authors interchangably, to describe this site on the the X chromosome.

Part C: Prevalence

In 1980, Herbst & Miller [15] reviewed four previously published surveys of families with X-linked mental retardation and found that 19 out of these 42 families demonstrated the fragile X chromosome. The authors used the British Columbia Health Surveillance Registry and obtained data, covering a 20 year period of time, on the number of families in which two or more siblings were found to have nonspecific mental retardation. They calculated the overall incidence of nonspecific X-linked

mental retardation to be 1.83 per 1000 males in the general population and estimated that of the fragile X syndrome to be 0.92 per 1000 males. Similarly in 1983, Fishburn et al. [16] restudied the brother pairs ascertained by one of the authors, Turner et al. [17], who in 1974, estimated the incidence of X-linked mental retardation in New South Wales to be 0.58 per 1000 males. Cytogenetic studies revealed that 12 of those 54 brother pairs demonstrated the fragile X chromosome and led to an estimate for the incidence of the fragile X syndrome of 0.13 per 1000 males.

In 1982 and 1983, eight large studies of the frequency of the fragile X chromosome in mentally retarded male populations were published [18,20-26] (See Table 1 at the end of this chapter). Many screened more than two hundred patients and although they used different selection criteria, they yielded reasonably similar results within the range of 1.7% [18] and 9.2% [20]. Four of the eight studies had results between 4% and 6% [21,22,24,25]. Two of the eight studies, Blomquist et al. [21] and Froster-Iskenius et al. [24] extrapolated the results from their institutions to the general population to estimate the frequency of the fragile X chromosome to be 0.4 and 0.5 per 1000 males respectively. The fragile X syndrome would then be second only to Down Syndrome in frequency as a recognized genetic etiology of mental retardation [27].

Part D: Phenotype

In the original reports of males with the fragile X syndrome no significant physical findings were noted [9-11]. Giraud et al. [10] commented only on a subjective impression of "large ears" in the affected males. Some authors, Jennings et al. [28] and Proops et al. [29], used the eponyms of the Martin & Bell Syndrome, Renpenning Syndrome, and marker X syndrome, synonomously with nonspecific X-linked mental retardation. Indeed, the family which was the subject of the report by Martin & Bell [7] was found to have the fragile X chromosome [30]. However, the family reported by Renpenning et al. [7] did not to have the fragile X chromosome upon retesting [31] and the possibility of a diagnostic phenotype appeared.

In the 1970's, reports in the literature by Escalante et al. [32], Turner et al. [33], Cantu et al. [34], and Ruvalcaba et al. [35] described a form of X-linked mental retardation that was associated with the feature of macroorchidism. In 1978, Turner et al. [36] restudied 16 families, some of whom were described in 1975 [33], for the presence of the fragile X chromosome and found it in six of these families. Furthermore, they found that all of the males with the fragile X chromosome had macroorchidism, while all the males with normal cytogenetic studies had normal testicular size. Reports with similar findings also were published in 1979 by Howard-Peebles et al.

[37] and Sutherland et al. [38].

This association between macroorchidism and the fragile X chromosome is far from absolute. In 1982, Brondum-Nielsen et al. [22] and Howard-Peebles et al. [39] in 1983, showed that a large proportion, 20% and 29% respectively, of the mentally retarded males in their institutions had macroorchidism. This was determined by using the standards of Prader [40], who established the 90th percentile of testicular volume in postpubertal males to be between 23 and 25 ml. and any volume above that was considered to be macroorchidism. Brown et al. [19] reported that four out of five males they tested with macroorchidism had the fragile X chromosome. Other reports did not find as close an association. Brondum-Nielsen et al. [22] found that only two of 52 patients with macroorchidism had the fragile X chromosome. They found within this group that two of the 11 patients with profound macroorchidism (>34ml) demonstrated the fragile X chromosome. They also tested all 52 males for other disorders known to cause mental retardation and macroorchidism. Howard-Peebles et al. [39] did find that nearly 25% of the mentally retarded males with profound macroorchidism had the fragile X chromosome, but, they did not test all the males in the 25-34 ml range. These frequencies, however, are much lower than those reported by Brown et al. [19].

In 1979, Jacobs et al. [41] reported on six families with

X-linked mental retardation. In five of these six families the males had macroorchidism. The males in the three families found to have the fragile X chromosome did not have macroorchidism and in the one family in which the males had macroorchidism the authors did not find the fragile X chromosome. Likewise, Herbst et al. [42] found that the feature of macroorchidism did not distinguish between the fragile X positive or negative males. Fryns et al. [23] found that only 6 of 19 patients with the fragile X chromosome had macroorchidism.

In 1983, Carpenter [43] in a review of the literature noted that macroorchidism was present in 88% of 120 postpubertal males with the fragile X syndrome, but, in only 23% of 34 prepubertal males in whom testicular volume was recorded. It would appear that macroorchidism is a reliable but not obligatory feature of the fragile X syndrome in postpubertal males and not a major feature in the young child. This is reflected in the current McKusick catalogue [44], in which males with X-linked mental retardation, fragile X positive, with or without macroorchidism are given the same classification number.

While much has been written on the feature of macroorchidism and the fragile X syndrome, other clinical findings have not received as much attention but deserve mention. Carpenter [43] reviewed and tabulated the clinical features described in the 252 affected males with the fragile X syndrome reported in the

literature. The IQ of the 70 affected males, in whom it was recorded, revealed that 40% had moderate mental retardation (IQ 35-49) and roughly 28% were in the mild (IQ 50-70) and severe (20-34) range. Only a few case reports in the literature [45-47] have claimed that the fragile X chromosome exists in males of normal intelligence. Carpenter [43] found that other physical abnormalities were noted in the males with the fragile X syndrome. This included the findings of ear abnormalities, strabismus, and abnormalities of the palate. Many other findings were also listed but at such low frequencies that they may be merely coincidental.

Part E: Cytogenetics

The cytogenetic techniques needed to study the fragile X chromosome have been understood for less than ten years. They are, however, now well accepted and described in the major cytogenetic texts and journals [49,50].

Seven years passed between the first description of the fragile X chromosome by Lubs [9] and the second report by Giraud [10]. In 1977, Sutherland [13] was able to explain this time lag on the basis of the lymphocyte culture media. He noted that expression of the fragile X site was obtained in only one of the commercially available media used by the cytogenetic laboratories

at that time.

In 1979, Sutherland [14] found that the media, "Medium 199", presumably the one used by Lubs [9] and Giraud [10], was somewhat deficient in thymidine and folic acid. The addition of relatively low doses of either inhibits the expression of the fragile X site. In 1981, Glover [51] and Mattei et al.[52] both showed that the inhibitory effects of folate could be overcome by methotrexate, a folate antagonist, but the inhibitory effects of thymidine could not. Methotrexate acts primarily by inhibiting the enzyme dihydrofolate reductase which is required for a one carbon transfer, necessary for the generation of the pyrimidine precursor, deoxythymidine monophosphate (dTMP), required for DNA synthesis [53]. This allowed recognition and confirmation by Glover [51] and Tommerup et al. [54] that thymidylate synthetase was one likely metabolic site involved in fragile X expression. They also discussed the employment of a direct thymidylate synthetase inhibitor, 5-fluorodeoxyuridine (FdU), as yet another method of producing the proper conditions for fragile X expression, in vitro.

Many culture variables may be involved in the detection of the fragile X chromosome [49] and different cytogenetic laboratories have their own preferred methods. The most important aspect of the lymphocyte culture conditions is to generate a thymidine depleted media. This must be done under

conditions that allow adequate lymphocyte growth and are reproducable test results.

Table 1

Prevalence studies of the fragile X syndrome in mentally retarded male populations.

Study author		Number	Prevalence	Feature(s)
Sutherland (1982)	[18]	444	1.7%	mental retardation
Carpenter et al.(1982)	[20]	65	9.2%	mental retardation family history
Blomquist et al.(1982)	[21]	96	4%	mental retardation
Brondum-Nielsen et al. (1982)	[22]	52	4%	mental retardation macroorchidism
Fryns et al.(1983)	[23]	250	3%	mental retardation family history
Froster-Iskenius et al. (1983)	[24]	242	6.2%	mental retardation
Kahkonen et al.(1983)	[25]	150	4%	mental retardation macroorchidism
Jacobs et al.(1983)	[26]	234	2%	mental retardation
This investigation		47	8.5%	mental retardation infantile autism

CHAPTER TWO: Infantile Autism

Part A: Definition

In 1943, Dr. Leo Kanner [55] published the original description of a group of children with severe developmental abnormalities and a unique quality of self-centeredness. He termed this syndrome, "early infantile autism." In an extensive review on the subject in 1981, DeMyer et al. [56] discussed the research that changed the consensus view of the syndrome. This disorder was originally viewed as a distinct entity. By the time of the 1977 meeting of the Professional Advisory Board of the National Society for Children and Adults with Autism [57], infantile autism had become recognized as a syndrome that could be diagnosed by a characteristic group of behavioral signs have a variety of associated features (i.e. mental retardation) and etiologies (i.e. phenylketonuria). (See Appendix A for the descriptions of these behavioral signs.) Much of this information was incorporated by the American Psychiatric Association as part of the latest edition of their "Diagnostic and Statistical Manual of Mental Disorders," DSM-III [58]. This was done to allow more consistency in diagnosis and to aid in future research on the subject.

There are six criteria (see Table 2) that are required for a person to be diagnosed as having infantile autism, according to

the DSM-III [58]. Two of the criteria, onset before 30 months of age and an absence of psychotic features, were included to distinquish infantile autism from the disorders of schizophrenia, mania and other forms of adult and childhood psychosis. Two other criteria are a severe lack of responsiveness to others with a failure to develop interpersonal relationships and bizarre responses to environmental stimuli. The former is the feature which Kanner [55] referred to as "autism" (See Appendix A: #2 and #4 for a more detailed description). Lastly, two criteria are related to disorders in many aspects of the development and use of language.

Table 2

DSM-III Criteria for Infantile Autism

A. Onset before 30 months of age.

B. Pervasive lack of responsiveness to other people (autism).

C. Gross deficits in language development.

D. If speech is present, peculiar speech patterns such as immediate and delayed echolalia, metaphorical language, pronominal reversal.

E. Bizarre responses to various aspects of the environment, e.g. resistance to change, peculiar interest in or attachments to animate or inanimate objects.

F. Absence of delusions, hallucinations, loosening of associations, and incoherence as in schizophrenia.

Part B: Prevalence

In review articles [56,59] the frequently cited prevalence figure for infantile autism is 4-5 per 10,000 children, although the DSM-III [58] uses a slightly lower figure of 2-4 per 10,000. The former figure was found by Wing et al. [60], in 1976. They reviewed in detail several large epidemiologic surveys on the subject. These were studies of either the general population or of children with a broad range of handicaps and most involved both written medical history and personal evaluation by the original authors. When these methods were used to establish the diagnosis of autism, there was remarkable agreement. One of the five studies used only medical histories and offered a lower prevalence figure. Although the studies predated the DSM-III [58], the criteria used appear to be essentialy the same. Thus, the prevalence figure of 4-5 per 10,000 is generally accepted.

Part C: Genetic Aspects

The possibility of genetic influences in infantile autism can be dated back to the original description by Kanner [55]. Indeed, he suggested that an "inborn error" would be needed to explain the early age of presentation in this disorder. Some 25 years later it was recognized that a 50-fold increased risk for developing autism existed for a child with an autistic sibling when compared to the general population [61]. Despite this fact,

reviewers questioned the data used to support the role of genetic influences in infantile autism, allowing for the possibility of rare mutations [62] or complex polygenic inheritance [62,63] which they could not exclude.

Twin studies have since provided strong support for a role of genetic influences in the development of infantile autism. In 1977, Folstein and Rutter [64] studied 21 sets of same-sexed twins in whom at least one met the then accepted criteria for autism. They found that four of 11 monozygotic twins were concordant for autism compared to none of ten dizygotic twins. This result was statistically significant at a p value of 0.055. Several years earlier Bartak et al. [65] had found that relatives of autistic children demonstrated a spectrum of cognitive and language defects, not limited to autism. Using this evidence, Folstein and Rutter [64] found that nine of 11 monozygotic twins were concordant for either a form of cognitive defect or autism compared to only one of ten dizygotic sets. This result was statistically significant at a much lower p value. Although a specific mode of inheritance could not be delineated, a genetic influence was suggested. In 1982, a group at U.C.L.A. headed by Dr. Edward Ritvo organized the Registry for Genetic Studies in Autism [66]. They reported recently [67] on 33 sets of monozygotic twins with all but one pair being concordant for autism. Among the dizygotic sets there were only three out of 24 concordant for autism. This strongly supports the findings

of Folstein and Rutter [64].

One often overlooked piece of epidemiologic data concerning infantile autism has been the consistent finding of a large excess of affected males. In 1975, Ando et al. [68] found the male to female ratio in children with infantile autism to be markedly different from that found in other forms of mental retardation. In 1981, Tsai et al. [69] cited no less than eight studies, between 1966 and 1979, that showed this ratio to be in the range of 3:1 to 5:1. In the same report they showed that females with infantile autism had more evidence of brain dysfunction and, more importantly, a higher frequency of affected relatives than those of the males with autism. They concluded that the females required "a higher dose of genes" to be affected with autism. Even though in 1973, Spence et al. [70] did not find the 4.77 to 1 ratio of their study to be compatible with a model of X-linkage, the work of Tsai et al. [69] suggests that at least a subgroup may be affected by this inheritance model.

Part D: Serotonin Levels

Many studies have been done throughout the years to study various biochemical parameters in patients with infantile autism. The finding of elevated levels of the neurotransmitter 5-hydroxytryptophan (serotonin) in peripheral blood of some patients with autism is interesting, although its significnace

remains unclear [71]. The original description was made, in 1961, by Schain et al. [72]. They studied a group of mentally retarded autistic children and found that six of the 23 patients had significantly elevated serotonin levels. Many years later Young et al. [71] of the Yale Child Study Center restudied this group after the removal of those patients with conditions known to be associated with hyperserotonemia or misclassified as autistic. The results still showed that a significant proportion of the autistic patients had elevated serotonin levels. This finding has been replicated in other studies [73,74]. There is virtually no information in the literature on serotonin levels in fragile X males or fragile X males with infantile autism.

CHAPTER THREE: The Fragile X Syndrome and Infantile Autism

Part A: X-Linked Mental Retardation and Verbal Disabilities

In 1974, Lehrke [75] found that a significant difference existed between the verbal and performance abilities in his group of males affected by nonspecific X-linked mental retardation. He postulated that the genes responsible for the X-linked mental retardation also explained their verbal disabilities. Similarly, Jennings et al. [28] had noted that over one-half of the published families with X-linked mental retardation were described as having significant or excessive verbal disabilities. Some of these families had the fragile X syndrome.

In 1980, however, Herbst [48] did not find any difference between the verbal and performance IQs in a population of patients with X-linked mental retardation. Recently, in a report to the International Workshop on the Fragile X and X-linked Mental Retardation, Chudley [76] discussed his results on the IQ testing of 29 males with the fragile X syndrome. Likewise, his group found only a small difference (five points) between verbal and performance IQ in this population. It would appear that the association between verbal disabilities and X-linked mental retardation has mainly an historical value.

Another area of speech-language function in fragile X males

has received attention. Some authors have claimed to recognize a distinct speech pattern [77-79], described as "repetitive jocular speech" [77], in males affected by the fragile X syndrome. These reports were of an anecdotal nature since little speech testing data were given. In 1979, Howard-Peebles et al. [80] studied the language deficiency in five families with X-linked mental retardation, including two families with the fragile X syndrome. They studied several aspects of language function and production. The results were notable for a generalized language deficiency and articulation defects similar to those found in other forms of mental retardation. Although the number of patients was small, they concluded that the "verbal studies employed showed little promise as a tool of differentiating X-linked verbal disabilities." Future studies of fragile X patients may someday provide answers.

Part B: Fragile X and Infantile Autism

In 1980, Turner et al. [77] noted in their study that one of the 25 males with fragile X syndrome had been diagnosed as autistic. However, Brown et al. [81] are given credit for first emphasizing an association of the fragile X syndrome and infantile autism some two years later. They reported four males with the fragile X syndrome who had features compatable with infantile autism, although not adhering directly to the DSM-III [58] criteria. All of the patients were ascertained because of

either a family history of mental retardation or the feature of macroorchidism. This report was soon followed by that of Meryash et al. [82]. They described a six year old boy with severe mental retardation, the fragile X chromosome, and behavioral criteria meeting those of the DSM-III [58] for infantile autism. This child was tested because of a family history of mental retardation. Three other such case reports can be found in the literature including: Brown et al. [83], in 1982, one additional male; Gillberg [84], in 1983, a set of monozygotic triplets; and August [85], in 1983, two brothers. Also in 1983, Levitas et al. [86], reported that autistic features were found in six of their ten fragile X males. The six met the DSM-III [58] criteria of either infantile autism or infantile autism, residual state. For the latter, he offered the results of psychometric testing done at the time of diagnosis to confirm the presence of autism. A two year old boy was diagnosed in this study because he had the feature of mental retardation or developmental delay. No mention was made of features such as macroorchidism or a family history of mental retardation that might have led the author to suspect the fragile X syndrome. Two other reports of large series of males with the fragile X syndrome have noted the presence of infantile autism in a significant proportion of their populations. Fryns et al. [23] in 1983, reported infantile autism in four of 16 males with the fragile X syndrome and Rhoads et al. [87] in 1984, in three of 17. These two studies noted the

occurrences of infantile autism but did not discuss the diagnostic criteria used. This makes the significance of these proportions difficult to evaluate.

Part C: Study Goals

Infantile autism is a disorder with a large number of etiologies, of which the fragile X syndrome has been implicated as one [67]. It is apparent that a subpopulation of patients with infantile autism show genetic influences [64,67] and the large excess of males with infantile autism is also well documented [69]. The idea that the fragile X syndrome with features of infantile autism could explain part of the male excess in infantile autism is straightfoward and was noted by Gillberg [84], in 1983.

In August, 1982, this study was begun to determine the prevalence of the fragile X syndrome in a population of mentally retarded autistic males. This study was the logical next step in evaluating a possible association of the fragile X syndrome and infantile autism and the extent of this association.

CHAPTER Four: Materials and Methods

Part A: Patient Selection

All patients in the study are from the Southbury Training School, a residential state institution with approximately 1,200 mentally retarded persons, located in Southbury, Connecticut.

A computer search of the medical records of the Southbury Training School revealed that 147 patients carried the diagnosis of infantile autism. There were 42 females and 105 males. The medical records of the males were examined in detail with regard to maternal, neonatal, and early childhood medical and developmental histories. This review was done to remove those patients who had another plausible explanation for their autistic features and to assure that the histories available were consistent with the DSM-III [58] criteria. This was the only requirement for entry into this study. The explanations for the exclusion of the males from the original computer list are in Table 3. The frequency distribution of the severity of mental retardation was similar for both groups and can be found in Table 4.

To meet the goals of the study it was decided that 40 to 50 autistic males should be screened for the fragile X syndrome. After the medical record review 48 males were selected. One patient was on placement and could not be located. Thus, a total

of 47 males were entered into the study. The chromosomal studies were considered part of the diagnostic work for the mental retardation and were requested by the physicians responsible for the mendical care at the Southbury Training School.

Table 3

Reasons for exclusion of cases (N=57) from this study.

		No. of Cases
1.	Prenatal infection	2
2.	Intrauterine maldevelopment	3*
3.	Injury at parturition	4
4.	Prolonged neonatal anoxia	4
5.	Major illness starting in neonatal period	7
6.	Major illness or injury prior to 18 months of age with subsequent change behavior	7
7.	Severe neurologic abnormalities	15
8.	History insufficient or incompatible	9
9.	Diagnosis of childhood schizophrenia	5
10.	Combination of above	1**

* - 2 of 3; pregnancies complicated by severe toxemia
1 of 3; child small for gestational age

** - combination of 3 and 5

Table 4

Frequency distribution of degree of mental retardation in the cases considered for this study.

Scores of Stanford-Binet IQ testing from medical records available from 98 of 105 cases.

(IQ range)		Borderline (85-68)	Mild (68-52)	Moderate (52-36)	Severe (36-20)	Profound (<20)
Included	*(N=45)	0	2	8	17	18
Excluded	**(N=53)	0	3	6	16	28
Total	(N=53)	0	5	14	33	46

* - Data available for 45 of 47 cases
** - Data available for 53 of 57 cases

Part B: Cytogenetic Techniques

Peripheral lymphocytes were obtained for culture in the following manner. Two tubes of 5 to 7 cc. of whole blood were drawn from each patient through a 21 gauge needle into heparinized vacutainer tubes. Transport time back to the laboratory was 2 to 6 hours. Approximately 2 cc. aliquots of whole blood were drawn out of the vacutainer tubes through a 20 gauge needle into two 3 cc. syringes. The red blood cells were allowed to settle and 0.3 cc. of the white blood cell/plasma suspension was placed in the appropriate culture media.

The lymphocyte culture media used for the fragile X testing was a low folate, low thymidine media that was capable of supporting lymphocyte growth and replication. These conditions were obtained by mixing 100 cc. of Medium 199 (GIBCO cat. #320-1153), 2 cc. of fetal calf serum (previously tested for allowing fragile X expression), 1 cc. of 200 mM L-glutamine (GIBCO cat. #320-5039), and 1 cc. of a Penicillin (5000 units/ml) - Streptomycin (5000 ug/ml) mixture (GIBCO cat. #600-5075). The pH of the final solution was in the range of 7.4 to 7.6. This solution was divided into 12 cc. aliquots and placed in 1 ounce "Brockway" glass bottles.

Lymphocytes were stimulated to undergo mitosis by the addition of 0.1 cc. of the mitogen, phytohemagglutinin (Wellcome Reagents Ltd.). The culture was then placed in an incubator at 37

degrees Centigrade and kept at a 45 degree angle with the caps tightly sealed in order to maintain the proper carbon dioxide concentration and pH. After 96 hours of growth, metaphase arrest was obtained by the addition of 0.12 cc. of colcemid (GIBCO cat. # 120-5211), which provided a final concentration of 0.01 ug/ml, 30 minutes prior to harvesting.

For harvesting, each culture was placed in a 15 cc graduated centrifuge tube and spun at a setting of 1000 RPM on a table-top centrifuge for 8 minutes. The pellet and approximately 0.1 cc. of supernatant were saved. The remainder of the supernatant was aspirated. The pellet was then resuspended and swelling of the cells was achieved by the addition of hypotonic (0.075M) KCl prewarmed to 37 degrees Centigrade. The first few drops were added slowly and a final volume of between 4 and 7 cc. was obtained. This method of swelling the cells aided in the later spreading of the chromosomes on the microscope slides. The harvested cells were returned to the incubator for 5 minutes and then recentrifuged. This and all other centrifugations were performed at the setting and time described previously. All but the pellet and an equal volume of supernatant were removed. Then 8 to 10 cc. of fixative, 3:1 methanol: glacial acetic acid by volume, was added and this was allowed to sit for 15 minutes. The fixative was made fresh and placed in the freezer before each use. Centrifugation was again performed and the supernatant removed. This fixation step was repeated until the pellet was

white and the supernatant clear. This was done to remove the red blood cell debris. The pellet was stored at -10 degrees Centigrade in 5 cc. of fixative in disposable glass tubes and capped with a cork.

To allow examination of the metaphase chromosomes from the lymphocytes, slides were prepared. Each box of new glass slides was cleaned and chilled in deionized water and stored in the refrigerator. The cells from the freezer were centrifuged and the supernatant removed. Then 0.1 - 0.2 cc. of the resuspended pellet was dropped onto a glass slide which was held at a 30 degree angle. The slide was then air-dried on a hot plate for 15 seconds and labeled. These microscope slides were made one week prior to staining.

Wright staining without trypsin pretreatment was performed on all slides. The slides were scanned at 125X and appropriate metaphase spreads then examined at 1250X. A count was made to assure that all 46 chromosomes were present in each spread. Then the unbanded chromosomes were closely examined for any abnormality in staining which could represent a gap, break, or other manifestation of a fragile site. All abnormalities were recorded. The presence of chromosomal breakage in greater than 10% of the complete metaphase spreads demonstrated that the proper culture conditions had been obtained. A minimum of 40 unbanded and 15 Giemsa banded diploid sets of chromosomes were

studied for the presence of the fragile X site. The Giemsa banding was part of the routine karyotyping done on all the patients in this study. This was done by other members of Dr. W. R. Breg's laboratory and will not be discussed in detail.

Any abnormality that was possibly a fragile X site was photographed and investigated further. The chromosomes were destained by placing the slides sequentially in the following solutions: 70% ethanol for 2 minutes; 70% ethanol for 2 minutes; 95% ethanol with 1% HCl for 2 minutes; and 100% methanol for 2 minutes. To allow better identification of the chromosomes they were banded by pretreatment with trypsin in a 0.9% NaCl solution. The slide was again stained with Wright's stain and the chromosomal spread in question reexamined at 1250X. The destaining, trypsin treatment, and Wright staining were sequentially repeated until positive identification of the chromosome was possible. Any patient found to have >4% of the lymphocytes expressing the fragile X site was restudied on an entirely new blood sample. This was done to establish the frequency of the fragile X expression and to confirm the diagnosis of the fragile X syndrome.

Some clinical data were obtained by other members of Dr. Breg's laboratory. Physical examinations, including anthropometric measurements, of the males diagnosed as having the fragile X syndrome was done. Information on family members of

these patients was obtained whenever possible and cytogenetic studies and genetic counseling offered.

Dr J. Leckman of the Yale Child Study Center conducted a pilot study on serotonin levels in males with the fragile X syndrome. This study tested a group of males with the fragile X syndrome, a group with infantile autism, and a normal control group. The patients from this prevalence study contributed to the first two groups and provided the only patients with both infantile autism and the fragile X syndrome.

CHAPTER FIVE: Results

A total of 47 males were selected for this study. Prior to the medical history review, seven autistic males from the original list of 105 had been tested for the fragile X syndrome by other members of Dr. Breg's laboratory. Four of these seven males were selected for inclusion in this prevalence study without regard to the prior cytogenetic studies, since they met the stated criteria. One of these four males had been found to have the fragile X syndrome. Of the remaining 43 males, three were found to have the fragile X site in >4% of their peripheral lymphocytes on two separate samples. In addition, one male demonstrated the fragile X site in one of 65 metaphase spreads, however, this did not meet the cytogenetic requirements of the fragile X syndrome. All other individuals were found to have no fragile X sites. All 47 males were found to have a normal 46,XY karyotype and a greater than 10% rate of non-specific background chromosomal breakage, an indication of proper culture conditions. The four patients found to have the fragile X syndrome were case records X-10, X-57, X-77, and X-123. One example of the fragile X site from each patient may be found in Figures 1 - 4.

The clinical features found (See Table 5) in these four males were typical of the phenotypic abnormalities reported in adult

males with the fragile X syndrome. The examiners felt that these patients had long narrow faces and protruding ears. On objective examination all four males had significant macroorchidism and large ears. In one case the macroorchidism was unilateral. They were all mentally retarded with a range from mild to profound. Only one of the four was found to have a family history of mental retardation.

Dr. Leckman (personal communication) measured whole blood serotonin concentrations in a total of 17 males with the fragile X syndrome. Overall, 18% or three of these 17 males had hyperserotonemia. As was expected, 38% of the autistic group and 7% of the control group had hyperserotonemia. The four autistic fragile X males had a wide range of serotonin levels with one being elevated. The numbers were too small to draw any conclusions, but, the feature of autism in the fragile X male did not appear to be related to any distinct whole blood serotonin level.

Table 5

Fragile X Patients

		X-10	X-57	X-77	X-123
	Age (years)	36	34	31	41
	% Fragile X	32	68	56	32
	IQ (Stanford-Binet)	33	56	32	20
*a	R. Testicular Vol.(ml)	34.6	45.3	74.2	50
*a	L. Testicular Vol.(ml)	19.7	50.2	58.6	38.5
*b	Mean Testicular Vol.	27.1	47.8	67.4	44.2
*c	Ear length (R/L)(mm)	70/70	72/70	75/72.5	75/75
*c	Ear width (R/L)(mm)	50/45	50/50	50/48	45/45
	Family History	yes *d	no*e	no*f	adopted

*a Length and width were measured.
Volume = (3.14/6) X (Length) X (Width) X (Width) [34]

*b = R. Testicular Volume + L. Testicular Volume X 0.5

*c Normal values for ear measurement in adult white males [88]:

Right Length	Mean 62.2	Standard Deviation 3.5
Left Length	Mean 62.4	Standard Deviation 3.5
Right Width	Mean 50.2	Standard Deviation 3.9
Left Width	Mean 50.2	Standard Deviation 4.2

*d See Appendix B for family pedigree

*e See Appendix C for family pedigree

*f The family did not respond to request for information.

Figure 1

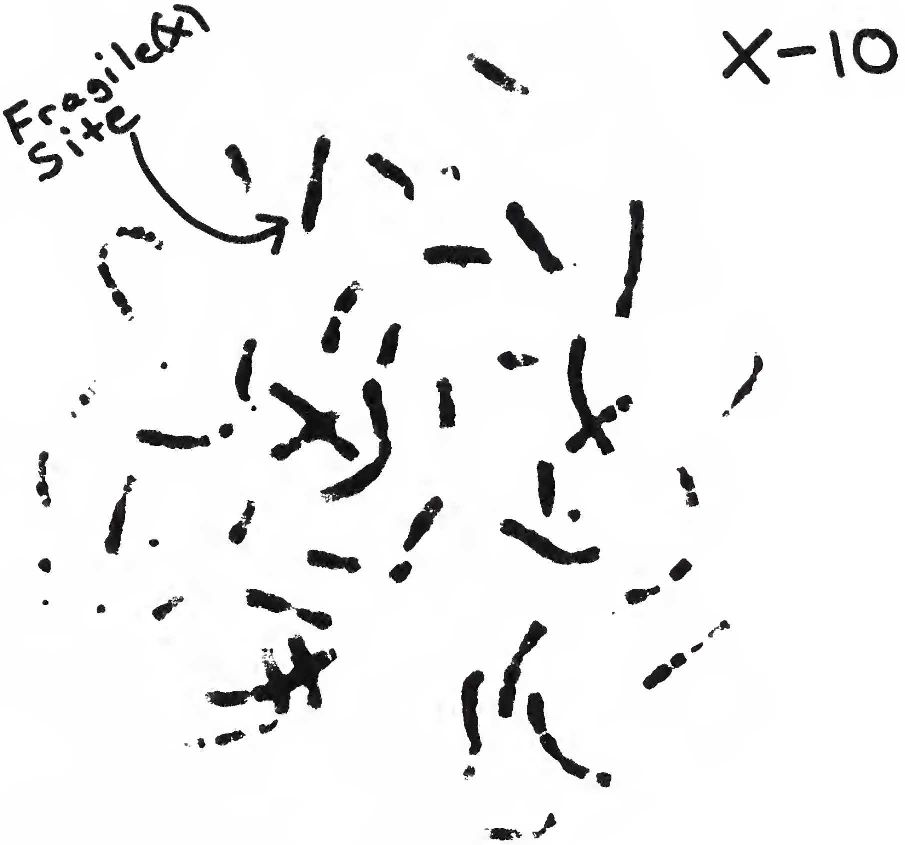


Figure 2

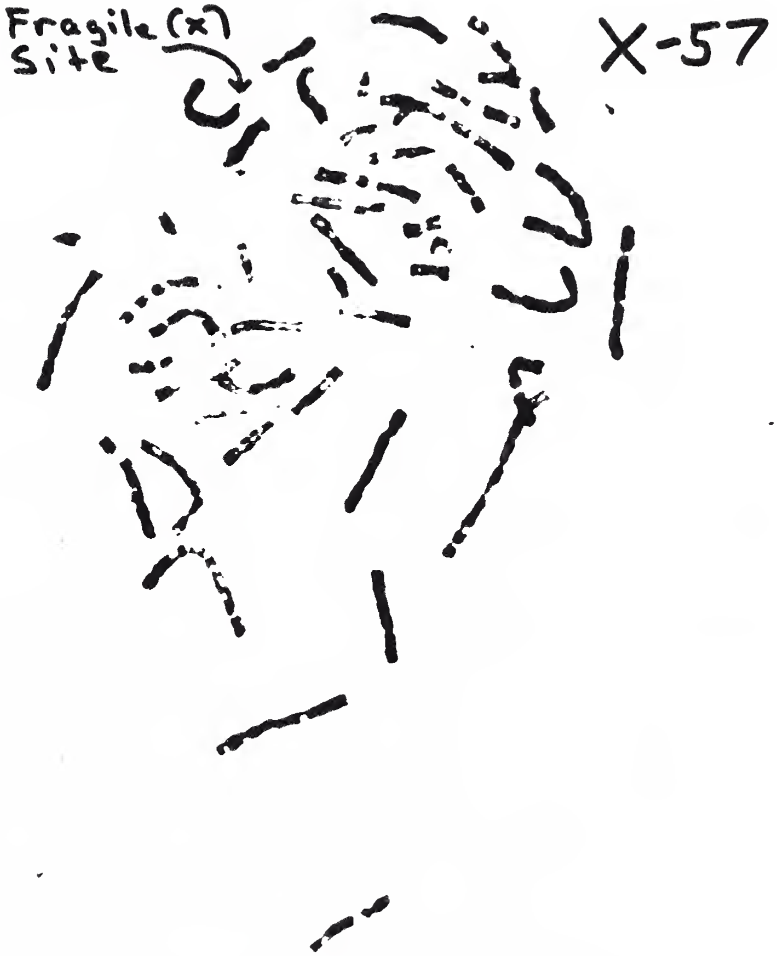


Figure 3

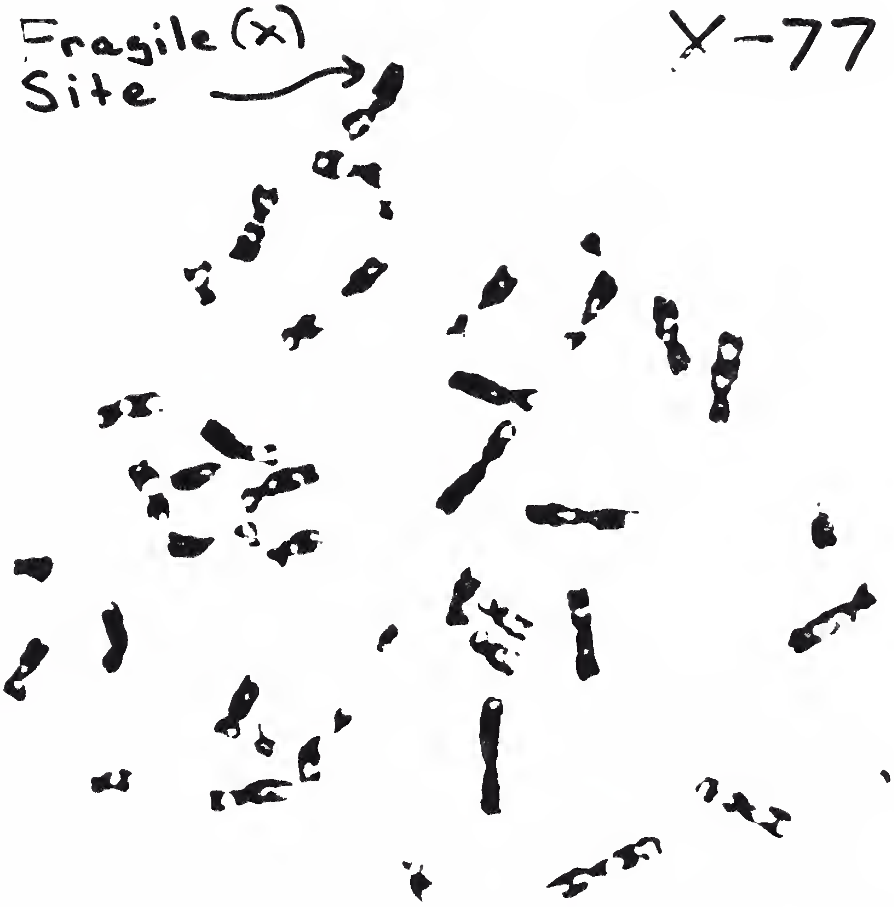
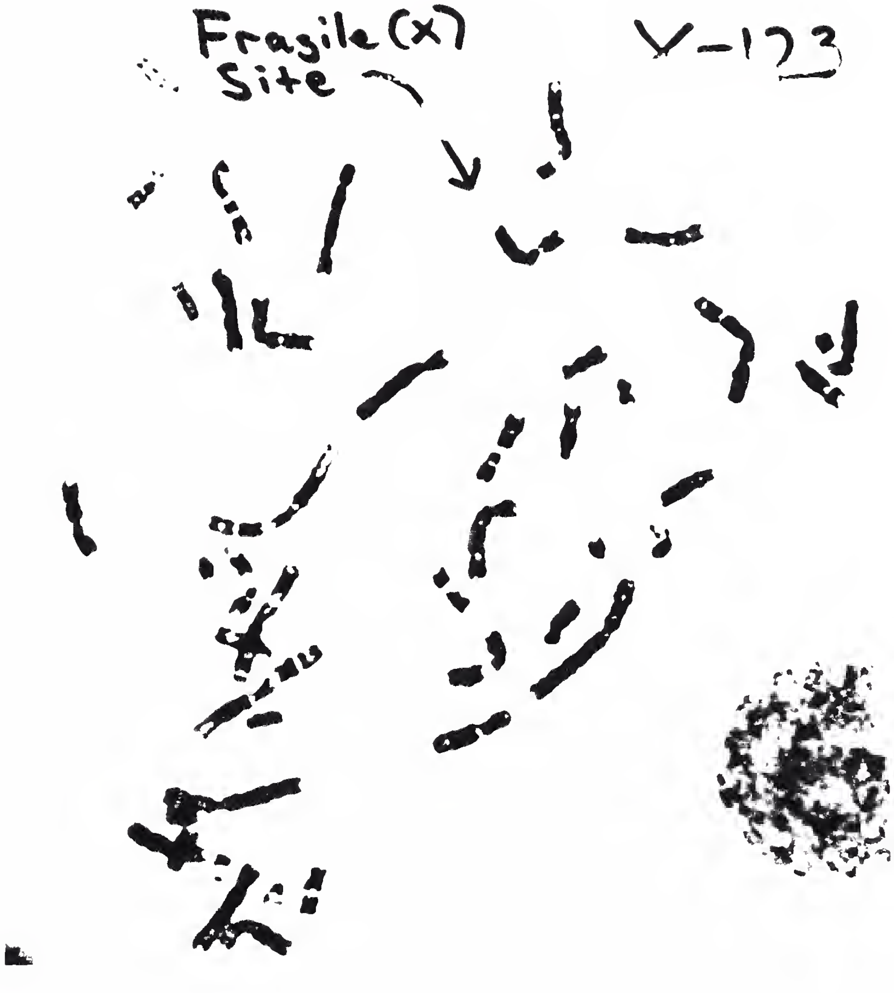


Figure 4



CHAPTER Six: Discussion

In some of the case reports which had found the fragile X syndrome in patients with infantile autism there was speculation that a significant frequency of association between these two disorders might exist [81,83]. This study was designed to provide prevalence data to evaluate the extent of this association. The results showed that four of 47 mentally retarded autistic males had the fragile X syndrome. This gives a prevalence of 8.5% in this population, which included seven autistic males in addition to those previously reported in the Southbury Training School group by us in 1984, Watson et al. [89]. That study is, to my knowledge, the only formally published prevalence study of the fragile X syndrome in a group with infantile autism and mental retardation.

Little other data are available to be used as as comparison to the 8.5% prevalence found in this group. Watson et al. [89] studied an additional 35 autistic males, not from the Southbury Training School, for the fragile X syndrome but found none. As a group they were less retarded than the group reported here, Watson (personal communication). Kahkonen et al. [25] reported that none of their 19 mentally retarded males with the diagnosis of infantile psychosis had the fragile X syndrome. The diagnostic term infantile psychosis has been one of many terms used for infantile autism [90]. Some of these patients may well

have met the DSM-III [58] criteria for infantile autism. The results of three unpublished reports by separate investigators who screened autistic males for the fragile X syndrome were reported to the International Workshop on the Fragile X and X-linked Mental Retardation by Leckman, Turner, Chudley and Mikkelson [76]. A total of three cases were found in the 106 autistic patients studied. No information was available on selection criteria or other features of those groups that could explain the higher prevalence found in our group studied at the Southbutry Training School.

A possiblity to be considered was that the prevalence found in this study was related only to the feature of mental retardation and was independent of infantile autism. All of the patients in this study were mentally retarded in addition to being autistic. However, the 8.5% prevalence of the fragile X syndrome found in this autistic, mentally retarded population was higher than the prevalence found in 7 of the 8 large studies [18,20-26] of mentally retarded males, but may well have included some who had the additional feature of infantile autism (See Table 1 at the end of chapter 1). Some of these studies used additional selection criteria such as macroorchidism [22] or a family history of mental retardation [20,23] to increase the likelihood of finding the fragile X syndrome. Still, only the study by Carpenter et al. [20] found a higher prevalence (9.2%). A study on the prevalence of the fragile X syndrome in a group of

mentally retarded males who lack the feature of infantile autism would provide a good comparison group. No study of this type has been performed, to my knowledge.

One possible explanation for the high prevalence of the fragile X syndrome in this report may be the method of patient selection. The patients studied here had no evidence by history, laboratory, or physical examination of an environmental or known biologic insult that could have explained their infantile auitsm. This may have increased the probability of a genetic etiology. A somewhat similar method was used by Herbst and Miller [15] to allow an accurate estimation of the frequency of X-linked mental retardation in British Columbia.

The results from this study demonstrated that when the additional feature of infantile autism was applied to a group of mentally retarded males it led to the finding of a higher prevalence of the fragile X syndrome. The number of patients in this study and the effects of different selection processes make the results of this study somewhat difficult to compare to others. However, the results of this study appear to be suggestive that an association between the fragile X syndrome and infantile autism exists in mentally retarded males.

A genetic influence in infantile autism has also been shown by the twin studies of Folstein & Rutter [64] and Ritvo et al. [67]. The recurrence rate for the development of autism amoung

siblings has been shown to be approximately 2% [61]. Although this rate is 50 times higher than in the general population, it might have been considered relatively small. For this reason, genetic etiology studies have not been thought to have a major role in the syndrome of infantile autism.

It is apparent from the results of this study and several previously published case reports [81-86] that the etiology for a proportion of males with infantile autism is the fragile X syndrome. This is a most important subgroup to identify for a number of reasons. The recurrence rate in this subgroup for the development of mental retardation and/or infantile autism is 50% for a male sibling and the improving capabilities of prenatal diagnosis [91] of the fragile X site may provide valuable information to a family. Also, early reports have been reviewed [92] and suggest a possible form of treatment or improvement with folate therapy, although this is not proven. Lastly, Levitas et al. [86] suggest that the autistic fragile X male may benefit from early intervention with special education.

There is a wide diversity of opinion regarding the use of fragile X testing for autistic males. Brown et al. [81,83] have recommended this testing for all males with autism of unknown etiology. They noted [83], however, that this might overwhelm the capabilities of many cytogenetic laboratories because of the number of people that would need to tested. The yield from such

a large screening would likely be very low but the advantages of such an approach are clear. For the many reasons noted earlier, this would allow the diagnosis of the fragile X syndrome to be made in those persons who may not have all its typical features. This is especially true for children who are too young to have the feature of macroorchidism, the severity of their mental retardation appreciated, or an affected brother. On the other hand, Meryash et al. [82] and August [85] advise fragile X testing for families with two or more autistic individuals and an X-linked pattern of inheritance. This would have a better percent yield then the policy of Brown et al. [83], but, would result in the medical community missing the diagnosis of the fragile X syndrome in many cases.

This study found the prevalence of the fragile X syndrome in a group of autistic mentally retarded males to be 8.5% and demonstrates that fragile X testing would be a valuable test in all males with infantile autism of unknown etiolgy who are mentally retarded. The population at the Southbury Training School represents but one part of the entire autistic population. It is probably rare for males with the fragile X syndrome to have a normal intelligence and macroorchidism is a reliable phenotypic feature in the adult [43]. Fragile X testing is therefore probably not necessary in the autistic postpubertal male unless they have the features of mental retardation or macroorchidism, with or without a family history of mental

retardation, although no study on this question has been performed. The question of whether or not to test every autistic male for the fragile X syndrome is difficult to answer because of the very early age that autism is diagnosed. At the present time, given the many potential advantages to the family and perhaps to the child for establishing the diagnosis, conservative medical management should be to test all male children with infantile autism of unknown etiology for the fragile X syndrome.

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Appendix A

National Society for Children and Adults with Autism: Definition of the Syndrome of Autism.

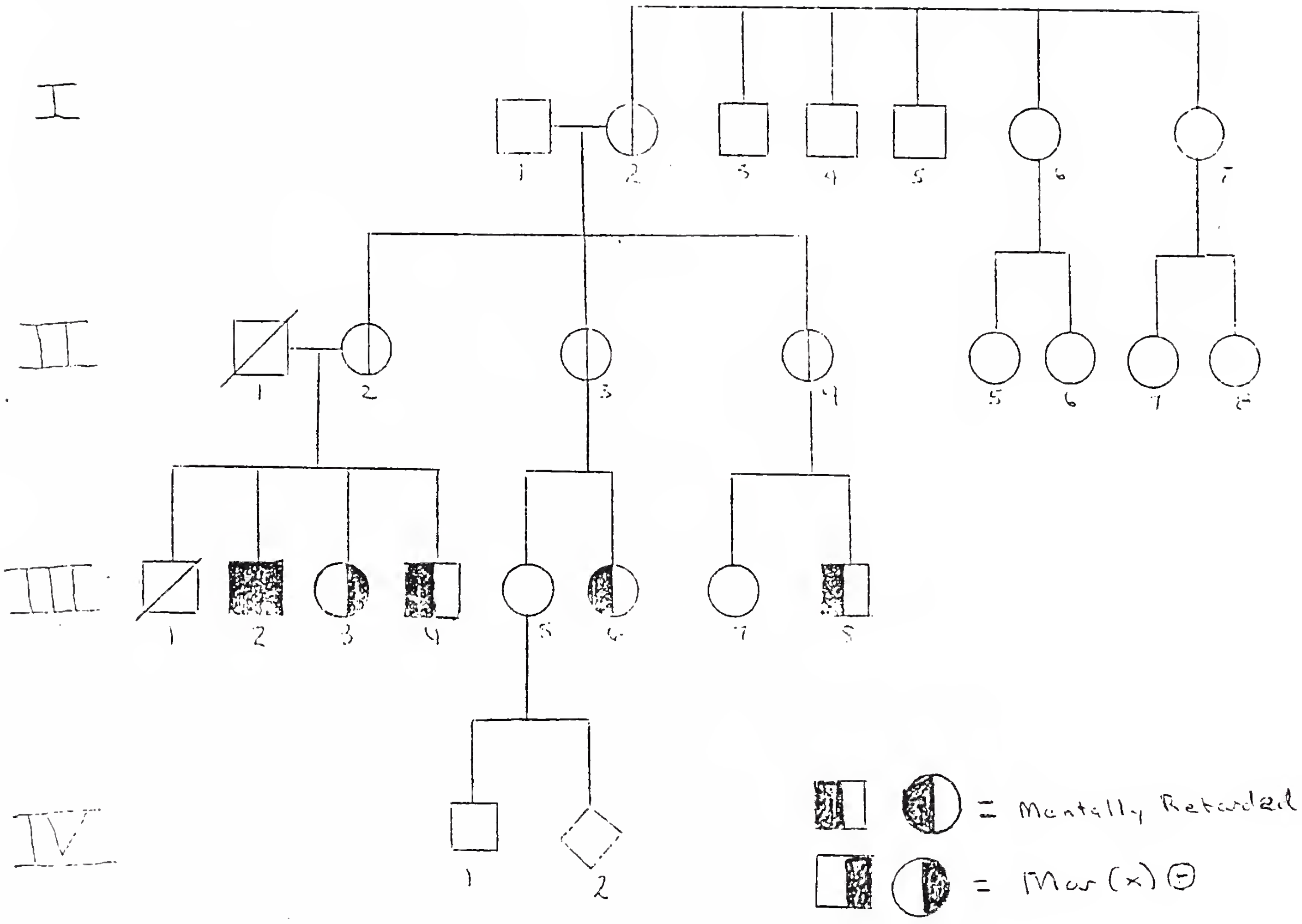
Essential features: Autism is a behaviorally defined syndrome. The essential features are typically manifested prior to 30 months of age and include:

1. Disturbances of developmental rates and sequences. Normal coordination of the three developmental pathways (motor, social-adaptive, cognitive) is disrupted. Delays, arrests, and/or regressions occur among or within one or more of the pathways a) within the motor pathway (eg, gross motor milestones may be normal, while fine motor milestones are delayed); b) between pathways (eg, motor milestones may be normal, while social-adaptive and cognitive are delayed); c) arrests, delays, and regressions (eg, motor development may be normal until age 2 when walking stops; some cognitive skills may develop at expected times, while others are delayed or absent; imitative behavior and/or speech may be delayed in onset until age 3, followed by rapid acquisition to expected developmental level).
2. Disturbances of responses to sensory stimuli. There may be generalized hyperreactivity or hyporeactivity, and alternation of these two states over periods ranging from hours to months. For example, a) visual symptoms. These may be close scrutiny of visual details, apparent nonuse of eye contact, staring, prolonged regarding of hands or objects, attention to changing levels of illumination; b) auditory symptoms. These may be close attention to self-induced sounds, nonresponse, or overresponse to varying levels of sound; c) tactile symptoms. These may be over- or underresponse to touch, pain, and temperatures, prolonged rubbing of surfaces, and sensitivity to food textures; d) vestibular symptoms. These may be over- or underreactions to gravity stimuli, whirling without dizziness, and preoccupation with spinning objects; e) olfactory and gustatory symptoms. These may be repetitive sniffing, specific food preferences, and licking of inedible objects; f) proprioceptive symptoms. These may be posturing, darting-lunging movements, hand flapping, gesticulations, and grimaces.
3. Disturbances of speech, language-cognition, and nonverbal communication. Symptoms may include: a) speech (eg, mutism, delayed onset, immature syntax and articulation, modulated but immature inflections); b) language-cognition (eg, absent or limited symbolic capacity; specific cognitive capacities such as rote memory and visual-spatial relations intact with failure to develop the use of abstract terms, concepts, and reasoning; immediate delayed, negative echolalia with or without communicative intent; nonlogical use of concepts; neologisms; c) nonverbal communication (eg, absence or delayed development of appropriate gestures, dissociation of gestures from language, and failure to assign symbolic meaning to gestures).
4. Disturbances of the capacity to relate appropriately to people, events, and objects, manifested by a failure to develop appropriate responsivity to people and assignment of an appropriate symbolic meaning to objects. For example, a) people: absence, arrests and/or delays of a smiling response, stranger anxiety, anticipatory response to gestures, playing "peek-a-boo," playing "patty-cake," and waving "bye-bye," reciprocal use of eye contact and facial responsivity, and appropriate reciprocal responsiveness to physical contact, a failure to develop a relationship with significant caretakers or an excessive reliance on caretakers. For example, caretakers may be treated indifferently, interchangeably, with only mechanical clinging, or with panic on separation. Cooperative play and friendships (usually appearing between ages 2 and 4) may not develop. Expected responses to adults and peers (usually appearing between ages 5 and 7) may develop, but are superficial, immature, and only in response to strong social cues; b) objects: absent, arrested and/or delayed capacities to utilize objects and/or toys in an age-appropriate manner and/or to assign them symbolic and/or thematic meaning. Objects are often used in idiosyncratic, stereotypic, and/or perseverative ways. Interference with this use of objects often results in expressions of discomfort and/or panic; c) events: there may be a particular awareness of the sequence of events and disruption of this sequence may result in expressions of discomfort and/or panic.

Taken from Freedman et al. [90].

Appendix B

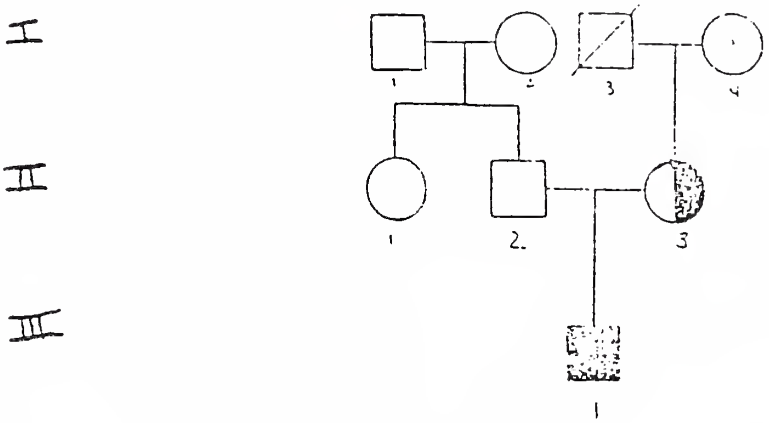
This is the family pedigree from patient X-10. The details from the pedigree may be found on the following page.



Individual ID	Sex	Age DOB	IQ	Mar(X) cells / total cells scored	Comments
II-2	F	1922	Normal	0/61	Declined retest
II-3	F		Normal	nt	
II-4	F		Normal	nt	
III-1	M				Reportedly Down S Seeking confirmat
III-2	M	1948	33	15/50	Autistic
III-3	F	1950	Normal	23/110	
III-4	M	1956		nt	Retarded; in an institution in New Jersey. Fragile X by hist
III-5	F		Normal	nt	
III-6	F	[illegible]	Dull	nt	
III-7	F		Normal	nt	
III-8	M		Retarded	nt	Fragile X by hist
IV-1	M		Normal	nt	

Appendix C

This is the family pedigree from patient X-57. The patients mother was found to be a carrier. There was no other family history of mental retardation or presence of the fragile X site.



Individual ID	Sex	Age	IQ	Mar(X) cells / total cells scored	Comments
I-3	M		---	nt	
I-4	F		Normal	nt	Unavailable
II-2	M		Normal	0/50	
II-3	F		Normal	5/100	Also mosaic for small marker
III-1	M	1950	IQ 56	91/150	Autistic

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